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Yang, Nan

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CHAPTER 1

General introduction

Introduction

Cervical cancer is the second most common cancer in women worldwide, with more than half a million new cases diagnosed in 2005. Most cervical cancers occur in developing countries (83%), mainly in Latin America, sub-Saharan Africa, and the Indian subcontinent (1). Cervical cancer is an important cause of early loss of life as it affects relatively young women. Cervical cancer is preceded by precursor lesions; cervical intraepithelial neoplasia (CIN). CIN I regresses in most cases, while up to 50% of the CIN III lesions could progress to cervical cancer when left untreated (2). It is estimated that the progression from CIN to cervical cancer generally takes a decade (3). Infection with high risk types of human papillomavirus (hr-HPV) is the main cause of cervical cancer (4). Several hr-HPV types have been identified of which HPV 16 and 18 are the most important, being responsible for 70% of all cervical cancer cases (5;6). The virus is acquired mainly through sexual activity (7). Risk factors associated with development of cervical cancer include sexual activity starting at a young age (< 16 years), a high total number of sexual partners (more than four), history of genital warts and immunosuppressive agents treatment (8). These risk factors are associated with acquiring and persistence of hr-HPV infections. The treatment of cervical cancer includes surgery, chemotherapy and radiotherapy. Surgery or chemoradiotherapy can cure 80-95% of women with early stage disease (stages I and II) and 60% with stage III disease (9-12). Early detection of precursor lesions and cervical cancer by cytomorphological assessment has been introduced in most countries and resulted in a significant decrease in the cervical cancer incidence. In addition, more patients are diagnosed with early stage cervical cancer or preferably CIN II/III. Because of high false positive and false negative rates, efforts have been made to improve cervical cancer screening by the use of hr-HPV DNA or some other new biological and molecular markers testing (13). DNA hypermethylation of cervical cancer specific genes is one of these novel biomarkers.

1 Precursor Lesions

1.1 histological and cytological classification

Cervical cancer is characterized by a well-defined pre-malignant phase that can be suspected on cytological examination of exfoliated cervical cells and confirmed on histological examination of cervical biopsies. These pre-malignant changes represent a spectrum of histological abnormalities ranging from CIN I (mild dysplasia, with dysplasia in the basal 1/3 of the epithelium) to CIN II (moderate dysplasia, with dysplasia in 2/3 of epithelial layer) to CIN III (severe dysplasia/carcinoma in situ, with dysplasia in more than 2/3 of the epithelial layer) (figure 1).

The Bethesda classification was developed to differentiate between lesions likely to progress (high-grade squamous intra-epithelial lesions or HSIL) and lesions less likely to progress (low-grade squamous intra-epithelial lesions or LSIL) to cervical cancer (15). The Pap classification was developed from 1941 (16). Table 1 shows the correspondence between the different histological and cytological nomenclatures.

1.2 Treatment of precursor Lesions

Although most of CIN I lesions are associated with many types of HPV, the distribution of high-risk types in CIN I lesions is different from that seen in CIN II/III lesions and CIN I lesions can be primarily associated with low-risk types of HPV (17). In the Netherlands, all CIN II and CIN III lesions are treated, because it is still impossible to identify those lesions that are most likely to progress. CIN I lesions are left untreated, because most of these lesions will spontaneously resolve and development from CIN I to CIN II/III and cancer is generally presumed to take 5-10 years. Treatment options for CIN II and CIN III are large loop excision of the transformation zone (LLETZ), cryocoagulation, laser evaporation, cone biopsy or hysterectomy, depending on the preference and expertise of the gynecologist. LLETZ is the preferred treatment, since this can be performed in an out-patient setting and allows histological examination of the removed tissue, while fertility is preserved and more than 90% of patients are cured after the procedure (18). After diagnosis of CIN I, patients return 6 months after diagnosis for a Pap smear. Follow-up after treatment for CIN II or CIN III consists of Pap smears taken 6, 12 and 24 months after diagnosis. If one of these smears is abnormal, a follow-up colposcopy is warranted.

2 Treatment of cervical cancer

The International Federation of Gynecology and Obstetrics (FIGO) staging system is the most commonly used (19). Squamous cell carcinoma accounts for about two thirds of all cervical cancers and adenocarcinoma is found in 15-25% of cases. Choice of treatment is based almost entirely on FIGO stage. Micro-invasive cancer can be treated by LLETZ or cone biopsy, if preservation of fertility is required. Early stage tumors can be managed by radical hysterectomy plus pelvic lymph node dissection or (chemo)radiotherapy. Advanced stage tumors are almost always treated by (chemo)radiotherapy (20). Five year survival approaches 100% for patients with tumors of stage IA and averages 70–85% for those with stage IB1 and small IIA lesions. Survival for more locally advanced tumors (stages IIB2 to IV) varies and is influenced significantly by the volume of disease, the patient's age and comorbidities. Overall, five year disease-free survival is 50–70% for stages IB2 and IIB, 30–50% for stage III and 5–15% for stage IV (8).

3 Cervical screening

European Union guidelines on cervical screening recommend that cervical cytology should be part of an organized population-based screening program beginning between 20–30 years and continuing until at least 60 years, with an interval of 3 to 5 years. Organized cervical cytological screening has been shown to reduce cervical cancer incidence and mortality by 70–90% (21). However, disadvantages of the current screening method include the low sensitivity and low positive predictive value leading to a delay in the diagnosis of cervical cancer and in an overshoot of diagnostic procedures (22). In all current cervical cancer screening approaches, liquid based cytology and more recently hr-HPV DNA detection are the most common recent improvements. There are also some new, less well validated screening approaches, such as

quantitative cytochemistry, detection of loss of heterozygosity (LOH) and DNA hypermethylation analysis (23).

3.1 Cytology

For a conventional Pap smear, the cervix is scraped with an Ayers spatula and/or cytobrush. The collected material is directly smeared on a glass slide and fixed. For liquid based cytology, cervical cells are collected by scraping the cervix with a sampling device made of plastic and the cells are then dissolved in a fixative liquid. Sensitivity of cytomorphological assessment ranged from 30% to 87% and specificity ranged from 86% to 100% (24). Both tests, conventional Pap smear and liquid based cytology, do not differ in sensitivity and specificity (25). However, liquid based cytology is getting introduced more and more, as the cytological assessment is quicker and automatic screening is feasible and additional molecular-based testing is possible, although the assay is more expensive (25).

3.2 hr-HPV DNA detection

HPV plays an important causative role in cervical carcinogenesis. HPV-16 accounts for 54% of the cervical cancer cases worldwide. The second most frequent HPV type is HPV-18 (15.9%) followed by HPV-33 (4.3%) and HPV-45 (3.7%). In patients with high-grade lesions, the most common HPV type is 16 (45.4%) followed by HPV-31 (8.7%) (26). Expression of the viral proteins E6 and E7 is pivotal for cervical carcinogenesis because E6 and E7 facilitate increased degradation of two important cellular regulatory proteins, p53 or pRB, respectively (27). HPV can be detected in cervical smears from almost all HSIL lesions and cervical cancers (28). The high sensitivity and hence negative predictive value of HPV DNA testing in a triage situation for women with low grade cytological abnormalities make this an effective test, and, especially when used as a reflex test on a liquid-based cytology specimen, very efficient. Women who are HPV negative can be reassured, avoid further follow-up and will re-enter the population-based screening program. The total HPV DNA positivity rose from 10.4% in patients with normal cytology up to 84.9% in women with HSIL and 87.2% in women with cancer (26). The management of women who are HPV positive without cytological abnormalities is less clear. Therefore, due to low specificity, especially in younger populations HPV testing alone will not sufficiently improve cervical cancer screening (6;29).

3.3 DNA methylation

In the past decade, abnormal patterns of DNA methylation have been recognized as frequent molecular changes in neoplasia (30;31). CpG islands in promoter regions of genes are targets for methylation, and if this occurs superfluously (referred to as hypermethylation), transcription may be blocked. Methylation of tumor suppressor genes contributes to an immortalized phenotype by silencing expression of genes responsible for control of normal cell differentiation and/or inhibition of cell growth. It is known to be an early event in carcinogenesis of many different tumor types (32). The detection of DNA methylation as novel biomarker in cancer research and diagnostics was revolutionized by two major discoveries. The first was the discovery of a very simple assay to visualize methylcytosine by treatment of genomic DNA with

sodium bisulfite. Bisulfite treatment results in the conversion of cytosine residues into uracil, except methylcytosine residues, which are protected against this treatment. By using, for instance, sequence analysis methylated and unmethylated DNA can thereby easily be distinguished (33). The second discovery was the development of methylation specific PCR. By taking advantage of the sequence differences within CpG islands of a promoter after bisulfite treatment, specific PCR primers can be designed that can distinguish methylated DNA from unmethylated DNA (34). An improvement of conventional MSP is real-time quantitative MSP (QMSP), which permits reliable quantification of methylated DNA (35).

In previous studies, several gene promoters were identified that are aberrantly methylated in cervical cancer as reviewed by Woodman *et al.* (36) and Wentzensen *et al.* (37). Using QMSP, we have previously demonstrated that 32 of 48 (67%) squamous cell cervical cancer (SCC) cases were methylated above the highest control (i.e. “hypermethylated”) for any of the gene promoters *APC*, *DAPK*, *GSTP1* and *MGMT* (38). In another study by our group, six (*CALCA*, *DAPK*, *ESR1*, *TIMP3*, *APC* and *RAR-β2*) out of 12 gene promoters analyzed were significantly hypermethylated in cervical cancer scrapings. Sensitivity of QMSP analysis with a four-gene panel consisting of *DAPK*, *CALCA*, *ESR1* and *APC* was 89%, which was equivalent to hr-HPV analysis and cytomorphology, but with a higher specificity (100%) (39). From studies of other groups, *SPARC* and *TFPI2* were identified as best discriminating between cervical cancers and controls. As both gene promoters were methylated in 20 of 22 cervical cancers and in only 3 of 21 controls (40). Steenbergen *et al.* investigated the pattern of methylation of *CADM1* (previously known as *TSLC1*) and found methylation in none of 21 normal or LSIL cases, compared to 7 of 20 (35%) HSIL and 30 of 52 (58%) SCC cases (41). Feng *et al.* demonstrated that *DAPK*, *RAR-β2* and *TWIST1* were increasingly methylated throughout cervical carcinogenesis, with 9 of 181 (5%) normal or LSIL cases methylated for at least one of these three gene promoters compared to 13 of 23 (57%) HSIL and 68 of 92 (74%) SCC cases (42). Cyclin A1 (*CCNA1*) was also identified as a gene specifically methylated in HSIL and cervical cancer, showing methylation in none of 38 normal or LSIL cases compared to 4 of 11 HSIL (36%) cases and 28 of 30 (93%) invasive SCC cases (43). Recently, Kahn *et al.* analyzed the frequency and relative level of promoter methylation for *DAPK1*, *CADM1*, *SPARC*, and *TFPI2* in residual liquid-based Pap tests of biopsy-confirmed HSIL, LSIL and cytologically negative for intraepithelial lesion or malignancy (NILM) patients. For each gene analyzed the frequency and relative level of methylation were increased in HSIL compared with NILM/LSIL samples. Methylation of each gene could distinguish HSIL from NILM/LSIL samples, but there was no significant difference in cumulative methylation in HSIL cases with histologic outcomes of CIN II versus CIN III (44). Lai *et al.* reported 6 genes (*SOX1*, *PAX1*, *LMX1A*, *NKX6-1*, *WT1* and *ONECUT1*) more frequently methylated in SCC tissues (81, 94, 90, 80, 78 and 20%, respectively) than in their normal controls (2, 0, 7, 12, 11 and 0%, respectively; $p < 0.0001$). Parallel testing of HPV and *PAX1* methylation in cervical swabs confers an improved sensitivity than HPV testing alone (80% vs. 66%) without compromising specificity (63% vs. 64%) for HSIL/SCC. Testing *PAX1* methylation marker alone, the specificity for HSIL/SCC is 99% (45).

The methylation profile of multiple genes can better distinguish cancer/HSIL from normal/LSIL samples. Although aberrant DNA methylation has the potential to function as a molecular biomarker of cancer/HSIL in liquid-based Pap tests, additional genes that are selectively methylated in HSIL are needed to improve the clinical performance of methylation-based tests.

Aim and outline of this thesis

Current cytomorphological based cervical cancer screening has some limitations due to relatively high false positive and false negative screening results. New technologies have been introduced to improve the current screening approach. The detection of DNA methylation as novel biomarker in cancer research and diagnostics has been developed in the last 20 years. Different techniques to detect hypermethylation are reviewed in **chapter 2**. The advantages and disadvantages of these techniques and the choice of methodology will be discussed.

Currently there are a number of gene promoters known to be methylated in cervical cancer. However, not much is known about methylation of cervical cancer precursor lesions. In **chapter 3** more insight is obtained in the course of methylation throughout cervical carcinogenesis using QMSP of nine gene promoters. Furthermore, to evaluate whether gene promoter methylation can be used to distinguish LSIL from HSIL lesions, DNA of paraffin embedded tissues from normal cervix (n=20), LSIL (n=20), HSIL (n=20), adenocarcinomas (AC) (n=20) and squamous cell cervical cancers (SCC) (n=40) was studied first because histology of the tissue is still considered as the golden standard. In addition, in cervical cancer patients we correlated promoter methylation with clinicopathological characteristics. Finally, QMSP of the same nine genes was performed to determine whether the methylation status of the underlying lesion was reflected in (55 available) corresponding cervical scrapings. Five of the nine gene promoters investigated had already been analyzed in a previous study (39), while the other four were suggested in recent literature to be specifically methylated in cervical cancer (40;41;43).

In order to find novel methylation markers our group recently performed a study by using pharmacological unmasking of cervical cancer cell lines followed by expression microarray in combination with a computational approach (46). This analysis revealed 45 potentially methylated genes of which 13 gene promoters appeared to be specifically methylated only in cervical cancer and not in normal cervixes as shown by BSP. In **chapter 4**, a more in-depth analysis of the methylation patterns of these 13 candidate genes in cervical cancer and normal tissue specimens was performed. Their possible relevance for the early detection of cervical cancer was evaluated in a large series of scrapings from patients with cervical cancer, low- and high-grade CIN and from otherwise healthy women.

Early recurrences after treatment (within 24 months) for HSIL might be due to inadequate treatment, while late recurrences (> 24 months) might represent de novo lesions (47). In **chapter 5**, we used QMSP to determine differences in gene promoter methylation in paired lesions from patients with early and late recurrent HSIL.

Screening programs have contributed to a decline of incidence and mortality of cervical cancer. However, women who do not respond to invitations for conventional smears in current cervical screening programs remain an important problem. These non-responders may be inclined to participate offering them a self-sampling technique. In **chapter 6** a feasibility study for the detection of DNA hypermethylation by QMSP in cervico-vaginal samples collected by a novel self-sampling device was performed. Methylation status, cytomorphology and hr-HPV positivity of cervico-vaginal samples obtained by a novel self-sampling device were analyzed and compared to data obtained from routine cervical scrapings collected from the same patients.

Finally, in **chapter 7**, summary and future directions are discussed in general, based on the results of the studies presented in this thesis.

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Table 1: Different nomenclatures for histological and cytological cervical abnormalities.

Histology		Cytology	
Dysplasia	CIN	Bethesda	Papanicolaou
Normal	Normal	Within normal limits	Pap 1
Benign atypia	Inflammatory atypia	Benign cellular changes	Pap 1
Atypical cells	Squamous atypia	ASCUS	Pap 2
Mild Dysplasia	CIN I	Low-grade SIL	Pap 3A1
Moderate Dysplasia	CIN II	High-grade SIL	Pap 3A2
Severe Dysplasia	CIN III	High-grade SIL	Pap 3B
Carcinoma in situ	CIN III	High-grade SIL	Pap 4
(Microinvasive) cancer	(Microinvasive) cancer	(Microinvasive) cancer	Pap 5

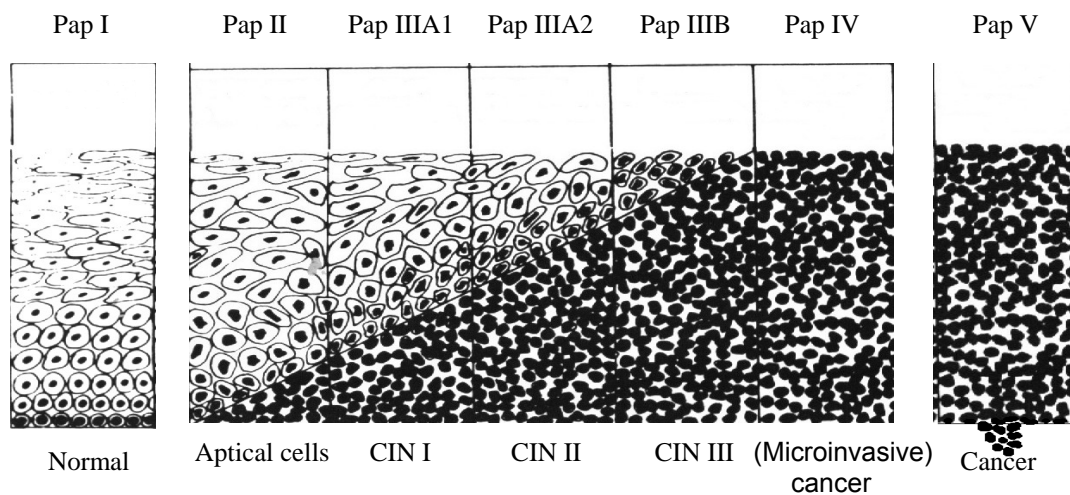


Figure 1: The development of CIN to cervical cancer (14).